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Model for the Motor Component of Dynein Heavy Chain Based on Homology to the AAA Family of Oligomeric ATPases

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Summary

Background: Recent iterative methods for sequence alignment have indicated that the 380 kDa motor unit of dynein belongs to the AAA class of chaperone-like ATPases . These alignments indicate that the core of the 380 kDa motor unit contains a concatenated chain of six AAA modules, of which four correspond to the ATP binding sites with P-loop signatures described previously, and two are modules in which the P loop has been lost in evolution.

Results: We report predicted structures for the six AAA modules in the β heavy chain of axonemal dynein, based upon their homology to a template of structurally conserved regions derived from three AAA proteins with experimentally determined structures (pdb:1A5T, pdb:1DOO, and pdb:1NSF). The secondary structural elements of the AAA modules in dynein correspond to regions of sequence that are relatively well conserved in different dynein isoforms. The tertiary structure of each AAA module comprises a major α/β N domain from which a smaller all-α C domain protrudes at an angle, as part of the putative nucleotide binding cavity. The structures of the six modules are assembled into a ring, approximately 125 Å in diameter, that resembles the structure of the dynein motor unit observed by electron microscopy.

Conclusion: The predicted structures are supported by procedures that assess global, regional, and local quality, with the module containing the hydrolytic ATP binding site being supported the most strongly. The structural resemblance of the dynein motor to the hexameric assembly of AAA modules in the hsp100 family of chaperones suggests that the basic mechanism underlying the ATP-dependent translocation of dynein along a microtubule may have aspects in common with the ATP-dependent translocation of polypeptides into the interior compartment of chaperones.

Introduction

Eukaryotic cells possess three distinct families of molecular motors, dyneins, myosins, and kinesins, that together generate the many different forms of cell motility that involve ATP-dependent translocation of the motor along appropriately organized elements of the cytoskeleton. Each of these families contains numerous different motors that are responsible for the various pathways of intracellular organelle transport that are required for metabolism and cell division, as well as being responsible for the changes in cell shape that determine the form of the organism during its embryonic development and enable the mature organism to move within its environment. Although the different motor proteins within a single family often have very diverse structures outside their motor domains, they are united in all possessing a defining set of highly conserved amino acid sequence motifs in their core "motor unit," the region of their polypeptide that is directly involved with ATP hydrolysis and force generation. These conserved sequence motifs indicate that all members of the family share a common inheritance of polypeptide folding in their motor unit and utilize essentially the same mechanism for converting the energy of ATP hydrolysis into mechanical work. Within each of the motor families, comparisons of sequence in the less conserved regions of the motor unit have clarified the evolutionary differentiation of the family into subfamilies that have different structural organizations and fulfill different cellular functions [1–3].

In broader evolutionary terms, the dynein, myosin, and kinesin families of motor proteins all fall clearly within the structural superfamily of P-loop ATPases [4, 5], but their relationships to each other and to other members of the P-loop superfamily are sufficiently distant that they are difficult to discern from amino acid sequence data alone. In spite of there being no significant similarity between the sequence motifs defining the myosin and kinesin families, as well as the fact that myosins interact with actin filaments and kinesins with microtubules, crystallographic findings have shown marked structural similarities among core motor units of these two families. The results of other detailed analyses are consistent with the two families having evolved from a common ancestor within the lineage giving rise to the present family of G proteins [6, 7].

The basic characteristics of the dynein family do not fit well into the same general pattern. Major differences include the much larger size of the individual motor unit (380 kDa), the presence of four equidistant ATP binding sites with P-loop motifs in the central region of the polypeptide of which only the most upstream site has hydrolytic ATPase activity [3, 8, 9], the location of its ATP-sensitive microtubule binding site on a 100 Å stalk projecting from the globular body of the motor [10–12], and the absence of the highly conserved residues found at critical positions of the nucleotide binding sites in both the kinesin and myosin families [7]. Although the resemblance of the sequence around the hydrolytic ATP binding site of dynein to that of the ATP-dependent

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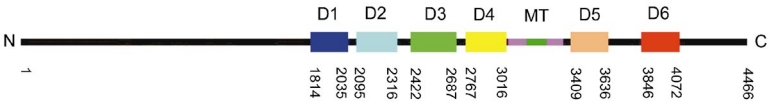

Figure 1. Schematic Architecture of the β Heavy Chain of Outer-Arm Axonemal Dynein

The six modules with AAA sequence motifs (D1–D6) are denoted by differently colored boxes. D1, closest to the N terminus, contains the hydrolytic ATP binding site. Amino acid residue numbers that delimit each of the six modules are indicated. The two predicted α-helical coiled coil segments (magenta) that form the stalk supporting the ATP-sensitive microtubule binding component (green) are also indicated.

protease ClpA had earlier been noted briefly [8], the relationship of the dynein motor to other P-loop ATPases remained obscure until Neuwald and coworkers [13] used new, more sensitive, sequence alignment protocols to detect the presence of six copies of the extended signature motif for the AAA class of chaperone-like ATPases within the motor region of the dynein heavy chain. These motifs were taken to indicate that the core structure of the dynein motor (Figure 1) comprises a concatenated chain of six AAA modules, each 35–40 kDa, of which the four closest to the N terminus of the heavy chain (D1–D4) correspond to the conserved ATP binding modules with P-loop signatures that had been described earlier [3], and the two closest to the C terminus (D5, D6) are modules in which the P-loop motif is presumed to have been lost in the course of evolution [13].

In this paper we describe models for the predicted structure of the six AAA modules in the dynein heavy chain derived from their sequence homology to the three AAA proteins with atomic level structures currently in the public database. Our results indicate that the secondary structural elements of the AAA modules forming the core of the dynein motor unit correspond to regions of relatively highly conserved sequence in different dynein isoforms from a broad selection of species. Important functional residues in dynein are identified as targets for future site-directed mutagenesis.

We selected the β heavy chain of outer-arm dynein from sea urchin for modeling, because the number and affinities of its ATP binding sites have been determined experimentally [14, 15] and because the isolated β heavy chain has been demonstrated to translocate microtubules in vitro [16, 17].

Results

The three proteins used as templates are: the hexamerization domain (NSF-D2) of the N-ethylmaleimide-sensitive membrane fusion protein (pdb:1NSF, [18, 19]), the δ′ subunit of *E. coli* DNA polymerase III, (Pol III δ′) (pdb:1A5T, [20]), and the regulating component of the ATP-dependent protease complex HslUV/ClpYQ (pdb: 1DOO [21]).

Assignment of Secondary Structures

The predicted structures for the six AAA modules in the dynein motor unit (D1–D6) are based upon the alignment of their sequences with those of the three AAA template proteins shown in Figure 2. In this alignment, the sequences of the β heavy chain of axonemal dynein that are used for modeling are indicated as D1A to D6A. The corresponding sequence regions of a typical cytoplasmic dynein are included for comparison (D1C–D6C). The arrangement of the three template proteins, HslU, Nsf-D2, and Pol III δ′, in the alignment is based upon their known three-dimensional structures. The arrangement of the dynein sequences is based upon that proposed by Neuwald and coworkers [13], and its validity has been confirmed by statistical analysis with PSI-Blast [22]. The color coding of the dynein sequences in each AAA module indicates the extent to which the residues in that module are conserved among different isoforms of dyneins from varied species.

The positions of the eleven sequence motifs that characterize members of the AAA superfamily [13] are indicated in Figure 2. The AAA motifs in dynein are most easily identified in the D1 module, where they are closest to the consensus in other branches of the AAA superfamily. In modules D2–D4, most of the AAA motifs are recognizable although they are frequently divergent. In the most degenerate modules, D5 and D6, the individual AAA motifs are difficult to discern, although the overall set of motifs is detectable. The AAA motifs in the template proteins can be mapped to regions of equal length in the dynein heavy chains with the exception of Box II and Sensor 2, which are represented by significantly shorter regions in dynein. Most AAA regions could also be mapped in modules D5 and D6, although the exact positions of certain motifs are subject to some uncertainty.

In module D1, the average conservation level of the residues included within the AAA motifs is 66%, whereas that of the nonincluded residues is only 44%. In modules D2–D6, the general level of conservation is lower, but the conservation of residues included in the AAA motifs remains substantially greater than that of the nonincluded residues.

Atomic Level Structure

The atomic level structure of the AAA-consensus region in the three template proteins, HslU (pdb:1DOO), NSF-D2 (pdb:1NSF), and Pol III δ′ (pdb:1A5T) is very highly conserved. In the final version of the template, in which 129 Cα atoms were superimposed from each of the three template proteins, the rms deviation over the structurally conserved regions (SCR) was 2.5 Å for 1A5T/1NSF and 3.6 Å for 1DOO/1NSF. The deviation over the phosphate binding loops was less than 1.0 Å RMS. In our predicted structures for the AAA modules in the dynein heavy chain, the fundamental structural framework, comprising the positions and orientations of the secondary structural elements included in the SCRs, is inherited from the templates. The conformations of the variable regions and the side chain packing, which are not determined by the templates, were obtained by conformational search, energy minimization, and molecular dynamics.

The structural models for each of the six AAA modules of the β heavy chain of axonemal dynein (Figures 3 and 4) consist of similarly sized regions averaging 236

residues around the nucleotide binding site. Each AAA module consists of a central β sheet made up of five parallel strands in the order 23415 and about eight surrounding α helices, depending on the module. The overall dimensions of each module are approximately 60 Å × 40 Å × 30 Å. The predicted α/β structure in dynein comprises a major N domain from which a smaller C domain protrudes at an angle as part of a nucleotide binding cleft or cavity. The N domain contains 4–5 helices surrounding much of the central β sheet, whereas the C domain consists of a bundle of three or four relatively short helices. The model rationalizes much of the observed pattern of sequence conservation among the various dynein sequences. Practically all conserved residues appear to be part of the α/β core of the model, whereas the least conserved regions all appear in the loop regions of the structure, consistent with the latter being isoform or species dependent. The topology and connectivity of tertiary folding in the dynein modules are unlike those in the other mechanochemical ATPases, kinesin and myosin [6, 7].

Comparison of the dynein modules (Figure 4) shows that the main secondary structural elements are present in all six and that the pattern of tertiary folding is highly similar, with the exception that the four-residue deletion in the Walker A motif of D5 shifts the position of the degenerate P loop by about 5 Å in this module. The minor structural differences that exist between the modules derive mostly from slightly different numbers of residues between SCRs. In addition, each module contains at least one variable loop greater than ten residues in length, and the positions of these probable surface loops are not conserved between modules.

The Putative Hexamer

Because most oligomeric AAA proteins, including two of our templates, NSF and HslU, occur as hexameric assemblies [13], we constructed a putative hexameric model of the dynein motor unit from the six individual AAA modules (Figure 5a–c). Preliminary analysis showed that the orientation and positioning of the individual AAA modules in the hexameric forms of NSF and HslU are almost identical, with an rms deviation of only 5.5 Å over the complete set of SCRs. This suggests that the hexameric organization is well conserved in different branches of the AAA superfamily and provides a suitable basis for assembly of the AAA modules in dynein. Modules D1–D6 of the dynein heavy chain were aligned into a hexameric arrangement by superimposing the eight-residue P-loop structure of each dynein module onto the P loops of successive modules around the hexameric structure of NSF. The proposed structures for the six dynein modules fit well in this arrangement and required only minor manual adjustment of one loop and several side chains to remove conflicts. This organization places helix H1 of each module with its axis tilted approximately 30° degrees to the plane of the hexamer and with its projection onto the plane, making one side of an overall hexagonal profile (Figure 5). The external diameter of the structure is 100–125 Å, the thickness is 40–60 Å and the diameter of the central channel is ~25 Å.

Each module appears securely lodged against the neighboring module. One contact area involves loops E2 to H3 and E3 to H4 in module (n) interacting with residues along one side of helices H4 and H5 in module (n + 1). A second contact area involves the C region of helix H8 in module (n) interacting with residues near the C end of helix H1 in module (n + 1). It is notable that this arrangement places both the N and C domains of module (n) in contact with residues in the N domain of module (n + 1), so that an ATP-induced change in the angle between the N and C domains similar to that described for HslU [21] could result in a functionally important alteration in the geometry of the hexamer. Many of the residues predicted to be involved in the interactions between modules are highly conserved among dyneins. For example, Glu-2218, Asn-2221, and Asp-2225 arranged along one side of helix H4 in module D2 are perfectly conserved (Figure 2), as are several residues along helix H5 in D1, D2, and D3.

The stability implied by the compactness of the proposed hexameric structure with its multipoint contacts between modules is consistent with the resistance of the dynein motor unit to proteolytic digestion under mild conditions. The only sites within the motor unit that are rapidly accessible to trypsin are Lys3238 and Arg3323 located within the microtubule binding site on its supporting stalk that projects out from between modules D4 and D5 [8]. The 215 and 124 kDa peptides that result from this cleavage presumably contain the D1 to D4 and D5 to C terminus fragments of the heavy chain, respectively [23]. Both peptides are resistant to further proteolysis at room temperature, with the former, but not the latter, being further protected by the presence of ATP.

In the hexameric model, the C terminus of each module is located approximately 30 Å from the N terminus of the next module, with a nearly unobstructed path between them. This distance can easily be spanned by the linker regions of 30 to 100 residues that join the six AAA modules in the intact dynein motor unit. Several of the linkers contain a small number of residues that are perfectly conserved among dyneins, suggesting that they play important functional or structural roles in the protein. However, there is no basis for modeling the structure of the linkers at this time.

ATP Binding Site

We identified the most probable site for binding of adenine nucleotide by exploiting the observations that only a few key residues anchor the nucleotide to its site in the template proteins NSF-D2 and HslU [18, 19, 21]. Using the *syn* conformation of ATP found in NSF-D2, we first approximated the binding position of ATP on the D1 module by using a rigid body electrostatic and van der Waals prescreen of the region close to the Walker A motif. The docking was then completed by local energy minimization using the full force field. This proposed ATP binding site lies near the base of the cavity between the N and C domains of the D1 module, close to one of the interaction regions with the adjacent D2 module. The location of this site in dynein is very close to that in NSF-D2 and HslU, although the ATP is angled slightly differently. When the SCRs of dynein-

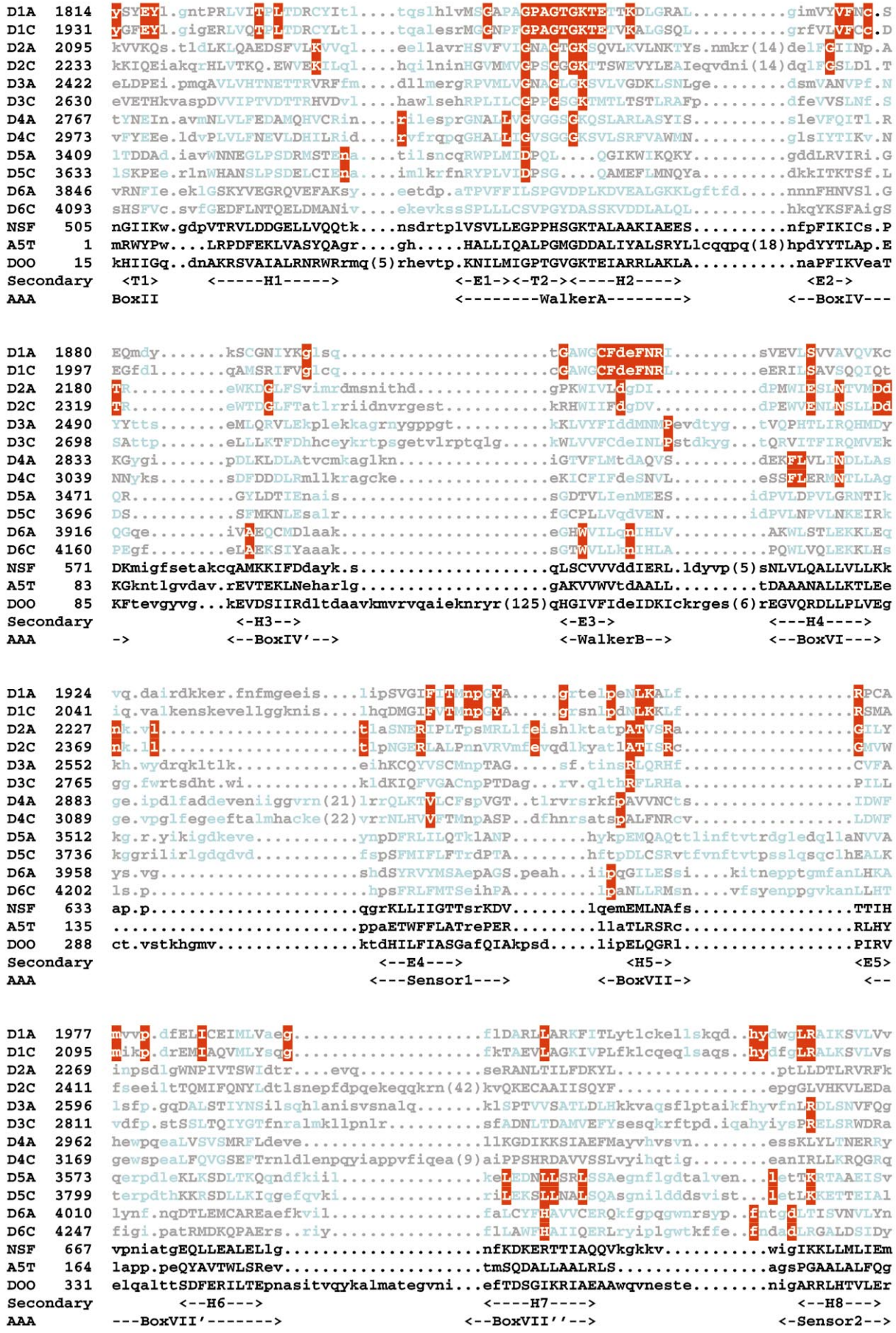

Figure 2. Multiple sequence alignment of the D1 to D6 modules of dynein heavy chains with AAA template proteins.

(D1A–D6A) The six AAA modules in the β heavy chain of outer-arm axonemal dynein from *Tripneustes gratilla*; (D1C–D6C) the six AAA modules in the heavy chain of cytoplasmic dynein from *Dictyostelium discoideum;* (NSF) the hexamerization domain of N-ethylmaleimide sensitive

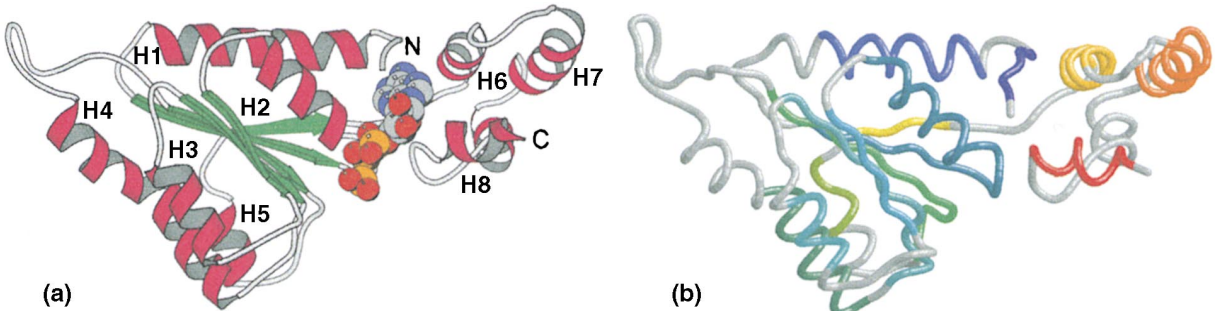

Figure 3. Molecular Model of the D1 Module of Outer-Arm Dynein β Heavy Chain

(a) View looking approximately parallel with the central β sheet showing the major α/β N domain and smaller protruding α C domain. A space-filling representation of ATP is present at the ATP binding site in the cleft between the two domains. N and C indicate the N-terminal and C-terminal ends of the D1 module.

(b) Backbone structure with positions of SCRs along the polypeptide indicated by the colored segments marking the locations of the corresponding AAA sequence motifs: violet shows Box II; dark blue shows helix 1; teal shows Walker A; sky blue shows Box IV; cyan shows Box IV′; dark green-blue shows Walker B; dark green shows Box VI; light green shows sensor 1; olive green shows Box VII; yellow and gold shows Box VII′; orange shows Box VII′′; red shows sensor 2.

D1 and NSF-D2 are superimposed, the positions of the γ-phosphates and adenine rings coincide within 0.5 Å and 3.2 Å, respectively.

The major part of the binding site in dynein is formed within the cleft in module D1 between the α/β core of the N domain and the four-helix bundle of the C domain. Residues from the N domain of module D2 complete the site and form a partial cover over the nucleotide. The site is formed in large part by the highly conserved regions GPAGTGKT (Walker A motif), GAWGCFDEFNRI (Walker B) [4], and SVGIFITMNPGY (Sensor 1), and by the moderately conserved region HYDWGLRAIKSVLV (Sensor 2), and the well-conserved residues in helices H4 and H5 of module D2 (Figure 2). The residues CFDEFNR in the Walker B motif are identical in the D1 module of all known dyneins and are considered the most characteristic fingerprint for dynein heavy chains [24]. The location of the ATP binding site suggests that the region connecting the N and C domains of the D1 module may act as a hinge and allow an ATP-dependent flexing between the domains similar to that observed in HslU [21] (see Discussion).

Because the positions of the residues interacting with the ATP are inherited from the templates, the extent to which these residues satisfy the biological criterion of being highly conserved can provide evidence for the validity of the sequence alignment used in the modeling. The residues predicted to lie within 5.5 Å of the phosphate chain of the ATP are shown in Figure 6. They include Thr1857 and Lys1858 from the Walker A motif, Asp-1904, Glu-1905, and Arg-1908 from the Walker B motif, Asn-1955 from Sensor 1, and Arg-2027 from Sensor 2, as well as Asp-2225 and Arg-2263 from the C ends of helices H4 and H5 of module D2. These residues are all fully conserved in the D1/D2 modules of all dyneins (Figure 2). The adenine ring lies within a partially hydrophobic pocket formed by the fully conserved Tyr-1814, Tyr-1818, and Glu-1817 near the N end of D1, together with fully conserved Glu-1860 from the P loop and Leu-2026 on Sensor 2 and a moderately conserved leucine at the C end of helix H1 of module D2. Although the adenine ring appears wholly enclosed, the 2′- and 3′- hydroxyls on the ribose point freely outward into the cavity, consistent with ribose-modified analogs of ATP being relatively good substrates for dynein [25, 26]. Although the detailed side chain configurations of the residues predicted to interact with ATP at the D1/D2 site should not be considered reliable, the highly conserved identity of these residues provides strong support for the validity of the underlying sequence alignment in these regions.

The general structure of a potential ATP binding site appears to be retained in modules D2–D6, although substantially fewer of the residues are highly conserved (Figures 2, 3, and 4). The absence of the usual conserved lysine in the Walker A motifs of D5 and D6 is consistent with lack of ATP binding in these modules [14]. A Walker B motif (Figure 2) is recognizable in all modules, but the paired acidic residues in this motif, which are present in most AAA ATPases and are required for functional activity in RuvB [27], are fully conserved only in module D1 (Asp-1904, Glu-1905); these residues are largely conserved in D3, but partly or completely absent in the other modules. The asparagine frequently found in the Sensor 1 loop of AAA ATPases is fully conserved in D1 (Asn-1955) and largely conserved in D3 (Asn-2529), but it

fusion protein; (A5T) δ′ subunit of the clamp-loader complex of *E. coli* DNA polymerase III; (DOO) regulatory component (HslU) of the ATP-dependent protease HslUV. Secondary, key elements of secondary structure conserved in the AAA template proteins: (H1–H8) helix 1 to helix 8; (E1–E5) strands 1–5 of the main β sheet; (T1, T2) turns 1 and 2 (the P loop); (AAA) characteristic sequence motifs of AAA proteins [13]. Uppercase letters show structurally conserved regions (SCR) derived from the three template proteins; lowercase letters show variable loop regions (see Methods). Colors indicate conservation level of residues in each of the six AAA modules in 20 dynein heavy chain sequences (five isoforms gathered from 14 species): red highlight shows perfectly conserved; blue shows conservation level above median for the module; gray shows conservation level below median for the module (see Experimental Procedures).

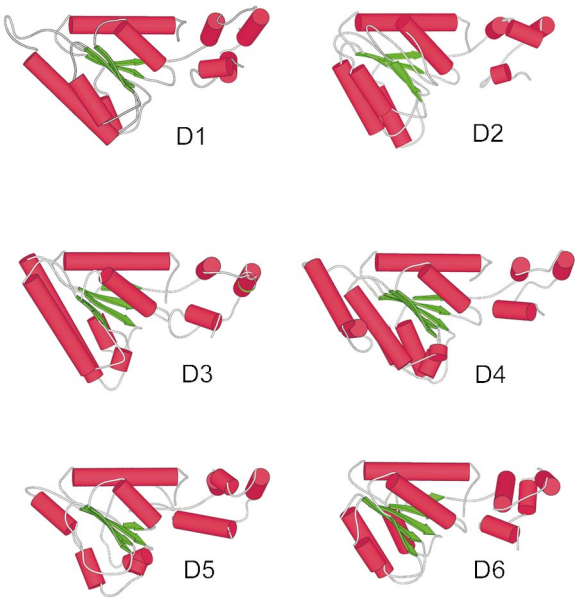

Figure 4. Structural Comparison of the D1–D6 Modules of Outer-Arm Dynein β Heavy Chain

The schematic (cylinder, arrow) representation highlights the generally conserved AAA configuration in the six modules, with local variations of the secondary structural elements in the individual modules.

appears absent from most of the other modules (Figure 2). The basic residues, Arg-2027, Arg-2661, and Lys-3628, identically located near the amino end of the Sensor 2 helices in D1, D3, and D5, respectively, are all fully conserved. A basic residue at this position is a well-conserved feature of AAA families (e.g., Lys708 in NSF-D2), and its position suggests that it may be involved in sensing the γ-phosphate moiety on the nucleotide [18, 19]. The overall pattern of residue conservation around the ATP binding sites in the AAA modules of dynein is consistent with ATP binding occurring in modules D1 through D4, but hydrolysis occurring only in D1 [14, 28]. However, the occurrence of low-level hydrolysis in D3, which has the next most highly conserved ATP binding site, is certainly possible, especially in cytoplasmic dynein.

Assessment of Model Quality

The global and regional quality of the models was assessed with the program Verify3D [29], which compares the environment of each residue in the structure to its average environment in a population of known proteins. The profiles obtained with the template proteins NSF-D2 and HslU varied between approximately 0.3 and 0.8 over the lengths of the modules. Because the low-scoring areas of the profile corresponded to structural regions known from the crystallographic structure to contact a neighboring module in the hexameric forms of the protein [18, 19, 21], we attribute the low scores to residues that are shielded in the physiological hexameric form but that become exposed to solvent in a separated module. The profile of the dynein D1 module showed a very similar variation, with the low-scoring areas in this case corresponding to the regions of helices H1, H3, H4, and H7. Because these are the regions predicted to contact the neighboring AAA modules in the predicted hexameric structure, this result provides good support for the validity of the models and their assembly into a hexamer. Profiles of the D2–D6 modules were similar to D1 but somewhat less regular. Scores averaged over the complete modules for D1, D2, D3, D4, D5, and D6 were 0.24, 0.12, 0.19, 0.21, 0.15, and 0.18, respectively, compared with 0.56, 0.45, and 0.47 for the template proteins, NSF-D2, Pol III δ′, and HslU. The somewhat lower average scores of the dynein modules (especially D2 and D5) compared to those of the templates are probably attributable partly to the additional exposed surface that becomes covered by the linker regions of polypeptide that join the AAA modules in the complete

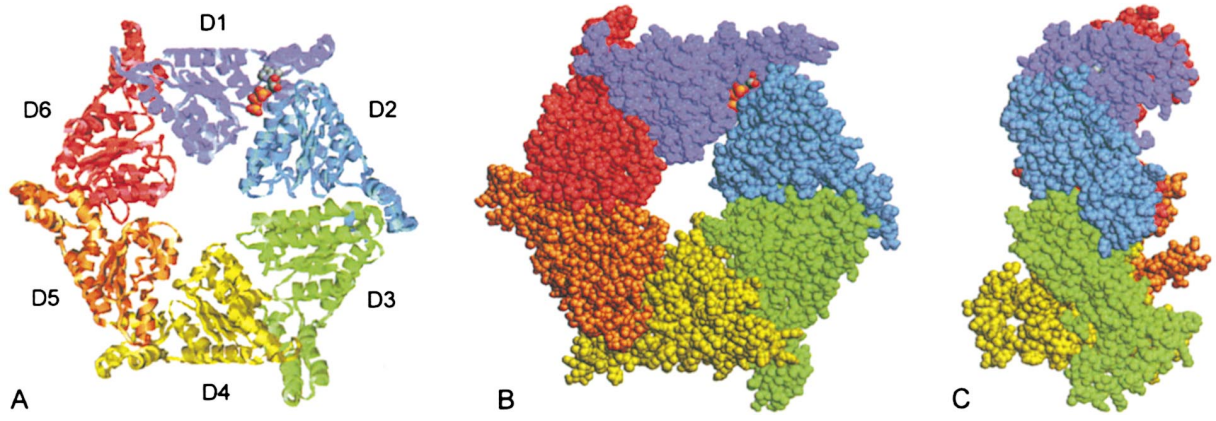

Figure 5. Putative Hexameric Organization of AAA Modules in the β Heavy Chain of Outer-Arm Dynein

The geometry of the hexamer is based on homology to the hexameric forms of NSF-D2 and HslU. The modules are colored according to their respective positions in the primary structure as shown in Figure 1 (N terminal in blue and C terminal in red). A space-filling representation of ATP, with CPK colors, is bound at the ATP binding site of the D1 module.

(a) Face view in ribbon representation.

(b) Face view in space-filling representation. The 2′ and 3′-hydroxyls on the ribose and the phosphate chain of the ATP remain visible.

(c) Same as (b) but in side view.

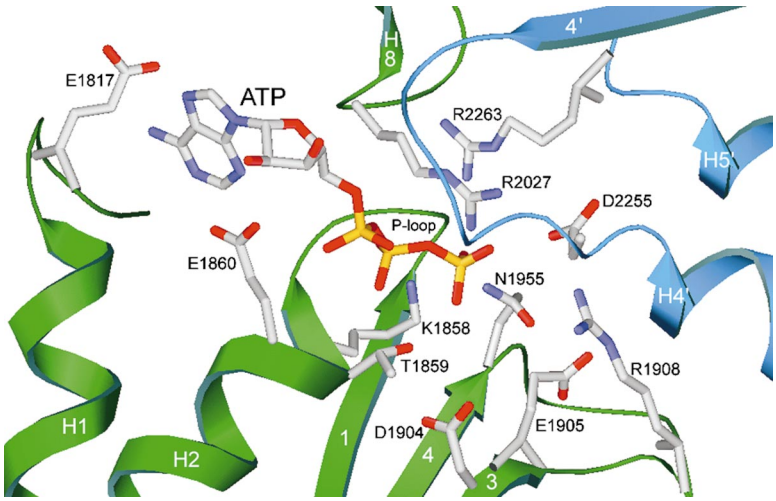

Figure 6. Schematic Representation of Residues at the ATP Binding Site in the Interface between Modules D1 and D2 of the β Heavy Chain of Outer-Arm Dynein

Helices H1, H2, and H8 as well as β strands E1, E3, and E4 of module D1 are shown in green: the P loop (Walker A) is the loop extending from E1 to H2; the Walker B motif is the loop extending outward from E3;. sensor 1 is the loop extending outward from E4; sensor 2 is the loop extending outward from H8. Helices H4′ and H5′ as well as strand E4′ of module D2 are indicated in blue. The side chains of residues located within 5.5 Å of the phosphate chain of ATP are represented in "stick" form, together with those of selected residues located close to the adenine ring. All the residues shown are perfectly conserved in the D1/D2 modules of 20 dynein heavy chains representing five isoforms gathered from 15 species. Three residues at the C end of helix H4′ are represented as coil to allow the residues below it to be seen. Conformations of side chains are shown for diagrammatic purposes and should not be considered reliable.

dynein heavy chain and partly to an accumulation of distributed small errors in modeling.

The local stereochemical quality of the models was assessed with the program PROCHECK [30]. In the present models the hydrogen bond values and Ramachandran plot positions fall within normal limits of comparison values in the databank, with 2%–5% of the residues in disallowed conformations. There are no significantly bad nonbonded interactions and no breaks in the peptide backbone. Two main-chain parameters, the peptide bond planarity and the α-carbon tetrahedral distortion, exhibit larger deviations, mostly as a result of distortion at splicing points between variable regions and SCRs.

Discussion

Dynein Motor as an AAA ATPase

The quality of structures obtained by homology modeling is principally dependent upon the choice of template and the validity of the alignment. Primary evidence for the dynein motor unit being a member of the AAA family is provided by the presence of six copies of the characteristic AAA pattern of sequence motifs within the sequence of the 525 kDa heavy chain polypeptide of dynein [13]. The mapping of the AAA motifs onto the dynein sequence has been confirmed in this work by a statistical analysis with PSI-Blast [22]. Support for the accuracy of the sequence alignment underlying the models is provided by the correlation observed between the conservation level of residues and their predicted importance to biological function. The finding that the residues predicted to lie within 5.5 Å of the bound ATP are completely conserved in the D1/D2 interface of all dyneins provides particularly strong support for the validity of alignment in the involved regions of these modules.

Our predicted assembly of the dynein modules into a hexameric structure is based upon hexameric forms of NSF-D2 and HslU. It is supported by the analysis with Verify3D [29], which confirms the probable regions of the dynein structure making contact with the adjacent modules, as well as by the moderate to high conservation of residues in the contact area between modules. The hexameric structure of the dynein motor unit is also supported by its resemblance to the dynein motor unit observed by electron microscopy [31].

However, there are differences in the degree of support for different modules in the predicted hexameric structure. Module D1, which is the best conserved among dyneins and also the closest to being a typical AAA ATPase, is the most strongly supported. On the other hand, the residues in the predicted C domains of D2 and D4 are relatively poorly conserved among dyneins, indicating a possible misalignment or a divergence of structures from the typical AAA form in this region.

Structural Organization of the Dynein Heavy Chain

When observed by high-resolution electron microscopy, the core of the dynein motor unit consists of a ring-shaped structure, approximately 135 Å in diameter, formed by about seven lobes surrounding a central cavity [31]. Apart from possibly having one additional lobe, this structure closely resembles the hexameric forms observed by electron microscopy in other AAA ATPases, including the hsp100/ClpA chaperones [32], the eubacterial proteasome equivalent (HslU/ClpY), the hexamerization component of N-ethylmaleimide-sensitive membrane fusion complex (NSF-D2), RuvB DNA helicase [27], and katanin [33–35]. We interpret six lobes of the dynein structure observed by Samso and coworkers [31] as corresponding to the six globular AAA modules in our hexameric assembly (Figure 5); the significance of the apparent extra lobe is discussed below. Direct assays of ATP binding have demonstrated the presence of four ATP binding sites in the β heavy chain of outer-arm dynein, one being a high-affinity site with a binding

constant of approximately 10^5 M^{-1} and three being lower-affinity sites with binding constants of approximately 10^4 M^{-1} [14]. The higher-affinity site presumably corresponds to the hydrolytic ATP binding site that has been localized to the D1 module from the location of its P loop close to or at the V1 site of vanadate-mediated photocleavage [8]. The low-affinity binding sites are putatively identified with the moderately conserved Walker motifs in D2, D3, and D4. The Walker motifs in D5 and D6 are highly degenerate, and these modules probably do not bind ATP. Because dynein ATPase activity decreases in direct proportion to the fraction of photocleavage in D1, it is unlikely that any of the other modules hydrolyses ATP at an appreciable rate [28]. Previous work has suggested that the ATP binding to the nonhydrolytic (D2, D3, and D4) sites may serve a regulatory function [36]. Consistent with the prime role of the D1 domain in containing the hydrolytic ATP binding site where the process of mechanochemical energy conversion is initiated, the sequences of the AAA motifs in this domain are more highly conserved among dyneins, as well as closer to those of other families of the AAA class, whereas the AAA motifs in domains D2 through D6 are more divergent, presumably as a result of weaker constraints in these regions of the dynein motor during evolution.

The seventh lobe present in reconstructed electron microscopic images of the dynein motor unit [31] may correspond to the base of the extended tail component that protrudes approximately 200 Å from the globular motor unit. This tail is formed by the portion of the dynein heavy chain on the N-terminal side of the D1 module, residues 1–1813 (Figure 1), and contains numerous short regions of predicted α helix [8, 9, 37]. An alternative interpretation is that the apparent seventh lobe might correspond to a highly degenerate seventh AAA module formed from the region of the heavy chain between D6 and the C terminus, residues 4072–4466. Interestingly, this region of the heavy chain does not appear essential to the function of cytoplasmic dynein, for although it is present in the cytoplasmic dyneins of animals as well as all known axonemal dyneins, multiple alignments show that it is absent from the cytoplasmic dyneins of the six fungi with available sequences. Biophysical characterization of HslU indicates that at least some AAA proteins can form a mixture of hexameric and heptameric assemblies [33], although the functional competence of the larger form is uncertain.

Structural Relationship of Dynein to Other Oligomeric AAA ATPases

The core structure of most oligomeric AAA proteins consists of an assembly of six subunits organized into a ring, approximately 120 Å in diameter and 50 Å thick, surrounding a central space of 15 to 20 Å. This hexameric assembly most often occurs as a regulatory component that facilitates the function of other more specialized components in a larger assembly, such as the SNARE complex that promotes membrane fusion in transport pathways and the internal protease chamber of eukaryotic proteasomes and their eubacterial equivalents [38, 39]. Cooperative interactions among the AAA modules in the core hexameric ring often play an essential role in the function of the assembly. In both proteasomes and RuvB helicase the inclusion of even a single inactive subunit into the AAA ring can be sufficient to disrupt the function of the complete assembly [27, 40], although in the chaperonin GroEL, which forms similar heptameric, but non-AAA-based, assemblies, these interactions appear limited to the immediate neighbors of an inactive module [41].

In the simplest form of hexameric AAA assembly, the six subunits are identical and monomodular (with a single AAA module per polypeptide subunit), exemplified by the microtubule severing protein katanin [35] and HslU [33], the six subunits are identical and each contains a single AAA module that serves both the ATPase and the structural/regulatory functions of the assembly through cooperative interactions between modules that regulate the steps in their ATPase cycle [42]. Other AAA proteins form into more complex assemblies that permit greater functional specialization of the individual AAA modules. One such form, found in both the eukaryotic proteasome and in dynein, has involved a six-fold replication of the gene encoding the polypeptide subunits forming the hexameric assembly. This replication permits the individual AAA modules to evolve separately and acquire distinct structural and functional properties. In the 19S regulating component of the eukaryotic proteasome, this differentiation of AAA modules has progressed to the point that the deletion of the gene encoding any one of the six is lethal, indicating that the individual subunits, although closely related in sequence, are functionally noninterchangeable [40].

In the dynein heavy chain, the fusion of the six AAA modules into a single polypeptide has permitted the hexameric assembly to evolve even greater structural and functional asymmetry, including the acquisition of the two substantial accessory structures that protrude from the motor unit. One of these acquired structures, formed from the region of the dynein heavy chain between D4 and D5, comprises the 100 Å stalk with predicted α-helical coiled-coil configuration that supports the all-important ATP-sensitive microtubule binding site [12]. The second is formed by the approximately 1800 residue extension onto the N terminus of D1 that comprises the tail component of the complete dynein heavy chain. The substantial α-helical component in these accessory structures suggests that they may both have evolved through a gradual extension and specialization of the much shorter coiled-coil/leucine-zipper structure often found in the N-terminal region of AAA proteins [43, 44]. Interestingly, the microtubule binding domain of katanin also involves an N-terminal extension from its AAA structure [35]. However, an alternative possibility is that the microtubule binding stalk evolved by prolongation of helices H6 and H7, which form a short leucine-zipper-like structure in the C domain of our present models, where it has been inherited from the NSF-D2 template.

Concomitant with the development of its asymmetrical structure, the dynein motor unit has evolved a functional dominance of the D1 module, which alone has retained the full ability for binding and hydrolysis of ATP [28]. Unlike the balanced extension of differential

function that appears to have occurred in the proteasome regulator subunits during evolutionary passage from archaebacteria to eukaryotes [39, 45], the other AAA modules in dynein heavy chains appear to have progressively lost much of their typical AAA function because they became fused into a single polypeptide. As discussed above, modules D2, D3, and D4 appear to have lost their capability for ATP hydrolysis, and the most degenerate modules, D5 and D6, show no significant binding of ATP. The partial loss of active function in five of its AAA modules notwithstanding, the dynein motor appears to require the presence of all its AAA modules for functional activity. Expression constructs lacking any of the AAA modules, or even the region of the tail component adjacent to D1 (residues 1400–1813), appear to show no ATPase activity [11, 46]. This requirement for structural integrity of the dynein motor unit resembles that of other members of the oligomeric ATPase class and may indicate that cooperative interactions of module D1 with the other AAA modules play an essential role in transferring the energy made available at the hydrolytic ATP binding site in D1 to the microtubule binding site attached between D4 and D5. Solubility difficulties have so far prevented determination of whether individual AAA modules of dynein are able to bind and hydrolyze ATP when expressed in vitro.

Because the large target size of very high molecular weight polypeptides, such as the dynein heavy chains, represents a relatively heavy developmental and evolutionary cost to the cell, such fusion of AAA modules into a single polypeptide has presumably become established only where it is of marked functional benefit. Most other large assemblages in cells, including ribosomes, proteasomes, type II chaperonins, and the chromosomal replication complex, are formed of numerous, relatively small subunits that have evolved with the capability for specific assembly into a complex structure. The only instance of a functionally active polypeptide that is comparable in size to the dynein heavy chain appears to be that encoded by the Saccharomyces gene YLR106C, which also contains six concatenated AAA modules in a single polypeptide of predicted mass 560 kDa [13]. Differences in the conservation of AAA motifs among the six modules of this gene suggest that one or two of its AAA modules have acquired a functional dominance resembling that of the D1 module in dynein although probably of lesser extent. Although the function of the YLR106C gene product is unknown at present, it appears to play an essential role in eukaryotes, for well-conserved orthologs are present in the genomes of Schizosaccharomyces, Arabidopsis, C. elegans, and Drosophila (I.R. Gibbons, unpublished observations). The functional characterization of this gene product may help to clarify the evolutionary relationship of the dynein motor to other members of the AAA class.

Biological Implications

The structural resemblance of the dynein motor to the hexameric assembly of AAA modules in the hsp100 chaperones and the regulator/translocator component of proteasomes and their bacterial equivalents [32, 39] suggests that the mechanism by which the energy released at the hydrolytic ATP binding site of dynein becomes transferred to the α-helical stalk supporting the microtubule binding site on the opposite side of the motor may be related to the ATP-dependent mechanism by which extended polypeptides become translocated into the interior compartment of chaperones and proteasomes. In HslU, the eubacterial proteasome regulator/translocator, the presence of ATP or a nonhydrolytic ATP analog at the nucleotide binding site results in flexing at a hinge region between the N and C domains, with a decrease of approximately 15° in the interdomain angle [21]. A similar flexing between the N and C domains of the enzymatically active D1 module in dynein may represent one of the initial events in the mechanochemical cycle coupled to ATP binding and hydrolysis in the dynein motor and would correspond to a local linear displacement of 10 to 15 Å within the D1 module. This displacement is substantially smaller than the observed step size of 80 Å with which dynein translocates along a microtubule [47]. Although up to about half of the necessary amplification of displacement can be accounted for by the extended lever arm that results from the attachment of the microtubule binding site between the D4 and D5 modules on the opposite side of the hexagonal assembly from D1, it is improbable that this can account for all of it. Additional amplification may be contributed by cooperative interactions of the hydrolytic D1 module with the nonhydrolytic D2, D3, and D4 modules that change the interdomain angles of the latter, possibly by modifying their affinity for binding ATP. Interestingly, the hexameric assembly of HslU shows evidence of negative cooperativity of nucleotide binding in adjacent AAA modules, with only three or four of the six able to bind a nonhydrolyzable ATP analog concurrently [21]. A similar cooperativity of ATP binding among the D1, D2, D3, and D4 modules of dynein [14] may contribute to the amplification of linear displacement, as well as underlie the demonstrated ability of the axonemal dynein motor to generate autonomous oscillatory motion when subjected to stretch activation. Such stretch-activated oscillation may be involved in the mechanism by which dynein generates oscillatory beating in cilia and sperm flagella [48, 49].

Overall, the predicted structures may be regarded as working hypotheses to stimulate new lines of investigation. For example, because the hydrolytic ATP binding site on module D1 is formed partly by structures in module D2, the in vitro expression of a construct that includes both the D1 and D2 modules may be more successful in producing an enzymatically active fragment than expression of D1 alone. Many of the residues predicted to interact with the bound ATP may prove interesting targets for future site-directed mutagenesis: in addition to the obvious targets in the Walker A and B motifs, these residues include Glu-1860, Asn-1955, and Arg-2027 in module D1 and Asp-2225 and Arg-2263 in module D2. However, a more complete structure of the dynein motor unit, including the regions outside the AAA core, will certainly be necessary to understand its function adequately.

While this paper was in final stages of completion a report appeared that discusses different aspects of dynein as a member of the AAA ATPase family [50].

Experimental Procedures

The three AAA ATPases used as templates for model building were the hexamerization domain (NSF-D2) of the N-ethylmaleimide-sensitive membrane fusion protein (pdb:1NSF, [18, 19]), the δ′ subunit of *E. coli* DNA polymerase III (Pol III δ′) (pdb:1A5T [20]), and the regulating component of the ATP-dependent protease complex HslUV/ClpQY (pdb:1DOO:chainE and 1DO0 [21]). The structures used for NSF-D2 and HslU both represent nucleotide-bound hexameric forms.

Homology-based computer modeling [51] was performed with the software programs Homology and Discover in Insight II (MSI Inc., San Diego, California). The hexameric structure was assembled and refined with the Swiss-PDB Viewer [52].

Sequence Alignment

The initial sequence alignment of the three template protein sequences was based upon a fitting of their Cα coordinates. The sequences of the putative AAA modules in the β heavy chain of outer-arm dynein from the sea urchin *Tripneustes gratilla* (gb:X59603 [8]) and of cytoplasmic dynein from *Dictyostelium discoidium* (gb:Z15124 [53]) were then added to the alignment as proposed by Neuwald and coworkers [13], with minor adjustments to allow connectivity between residues and to improve the alignment of consensus AAA motifs with regions of conserved sequence among dyneins of different species and isoforms. Because work during CASP3 [54] showed that alignments of distantly related proteins obtained from the iterative PSI-Blast server [22] are more reliable for structural purposes than those from obtained by pairwise methods, such as ClustalW, we validated our final alignment by comparing it to alignments of the dynein AAA modules with a variety of AAA protein sequences obtained from the PSI-Blast server [22]. The level of sequence identity in pairwise comparisons between the dynein and the templates is about 15%, approximately the same as that between pairs of the three templates.

Model Building

A structurally conserved region (SCR) of the templates was taken to be a segment of a single secondary structural element within which there was less than 0.8 Å rms deviation between the Cα coordinates for all paired combinations of the three templates. The three template structures, 1NSF, 1A5T, and 1DOO, were first compared pairwise and the SCRs of each pair identified. The set of consensus SCRs was then selected as those residues that met the SCR criterion in all paired combinations of the templates, except near the C terminus where the orientations of the two C-terminal helices in 1DOO differed from those of 1NSF and 1A5T and only the latter two were acceptable templates. After the SCRs had been identified in this way, the SCR coordinates in 1NSF were used as preliminary Cα coordinates for the segments of the dynein sequence corresponding to the SCRs.

For the variable loop regions of the dynein sequences, coordinates were taken from one of the template proteins that contained the appropriate number of residues wherever possible. When necessary, coordinates for a loop with plausible conformation were taken from a library of loops derived from proteins in the Protein Data Bank.

Model Refinement

The preliminary structure was refined using constrained energy minimization and molecular dynamic protocols. Peptide bonds at splice junctions were regularized first. The energy was then minimized by permitting mobility in the loop regions while holding the SCRs fixed. The energy of the SCRs was then minimized, before minimizing the energy of the loops. The few conflicting side chains that remained were cycled through a library of rotamers to find a low-energy conformation. Care was taken not to permit significant deterioration of inherited secondary structural elements during refinement.

Conservation Scores

A separate multiple sequence alignment containing 20 full-length sequences of dynein heavy chains, representing five isoforms gathered from 14 species, was prepared with the program ClustalX [55] with the Blosum 62 matrix [56]. The conservation level for each residue position in the this alignment was quantified by using the "quality score" of ClustalX with the Blosum100 matrix. The raw scores obtained in this way spanned a range of 60 to 100 and were expressed as a percentage by normalizing them to the range 0–100. This matrix was chosen to give a well-distributed set of scores when sample residues with different patterns of conservation were examined visually. Alignment positions containing one or more gaps were set to "blank" by the normalization process.

Supplementary Material

Information about the validation of sequence alignment, the Verify3D scores of dynein D1 module and template proteins (Figure S1), the multiple alignment of 20 dynein heavy chain sequences representing 5 isoforms gathered from 15 species (Figure S2), the amino acid conservation scores in AAA modules of the dynein heavy chain (Table S1), and the data bank references of the dynein sequences used in the alignment (Table S2) are available with the electronic version of this article at http://current-biology.com/supmat/supmatin.htm or upon request to the authors.

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